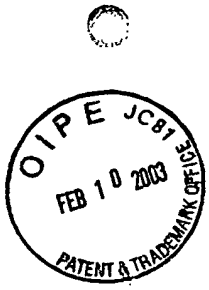

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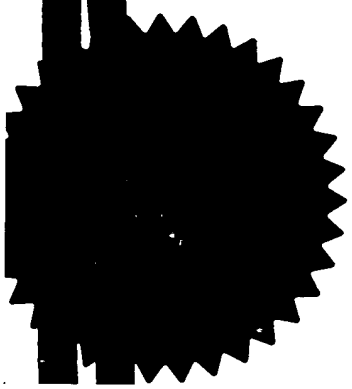

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1. Your reference — 24 SEP 1997

2. Patent application number *(The Patent Office will fill in this part)* — 9720184.2

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — SECTION 3(4) ACT) ECLAGEN LIMITED
BUSINESS CENTRE
23 ST MACHAR DRIVE
ABERDEEN AB24 3RY
APPLICATION FILED 22 9 97

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — INCORP. IN SCOTLAND

4. Title of the invention — BIOLOGICAL DNA LIBRARY SCREENING METHOD

5. Name of your agent *(if you have one)* — —

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — ~~As in (3) above~~
Kilburn & Strode
20 Red Lion Street
London
WC1R 4PJ

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
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BIOLOGICAL DNA LIBRARY SCREENING METHOD

The present invention relates to new screening methods to isolate genes encoding biologically active polypeptides from DNA libraries. In particular, the invention relates to a method for linking a biological phenotype to a gene.

There is considerable interest in "polypeptide display" methods for isolating polypeptides with useful phenotypes from gene libraries encoding a large mixture of polypeptides whereby the corresponding genes can be recovered swiftly and easilly. For example, there has been considerable success in identifying useful peptides or antibody fragments from DNA libraries using bacteriophage display methods to produce polypeptides fused to phage proteins which can bind to ligands of interest. In addition, there has been success in identifying useful polypeptides displayed on the surface of bacteria. More recently, there has been an interest in evolving new polypeptides displayed on bacteriophage and bacteria by successive rounds of gene mutation and selection for polypeptides binding to the ligand of interest. As an alternative to display of polypeptides on living organisms such as bacteriophage, cell-free transcription-translation systems have also been using whereby ribosomes are screened for binding to ligands of interest ("ribosome display") and whereby bound ribosomes are subjected to PCR to amplify associated mRNA molecules in order to determine the genes encoding polypeptides with binding activity.

Whilst display of polypeptides on microorganisms and transcription-translation methods have provided a link between a useful binding phenotype and genotype, these methods have largely been limited to detection of polypeptides by virtue of binding to a ligand such as an antigen or a chemical whereby the ligand is known and is usually available in a reasonably pure preparation. The identification of displayed polypeptides by virtue of binding directly to whole living cells has proven difficult. In many cases, the screening for binding phenotypes is a substitute for the isolation of biologically active polypeptides which display a useful biological phenotype upon interaction with target living cells. In such cases, it is necessary, having isolated the binding phenotype and determined the corresponding genotype, to then test the binding phenotype for biological activity. As only a proportion of polypeptides with a specific binding phenotype may provide the required biological activity, this phenotypic selection process is therefore inefficient and is reliant on a surrogate binding screen which may not select for biologically active polypeptides. In some cases where the ligand for phenotypic screening is not available or not known, then screening for new biologically active polypeptides may not be possible.

The present invention provides a new method to directly select a biological phenotype encoded by a DNA library and expressed via polypeptide display. The method provides a direct link between biological phenotype and genotype without the need for any prior knowledge of the binding phenotype of the displayed polypeptides. In one embodiment of the present invention, the stimulation (or inhibition) of a biological response by binding of displayed biologically active polypeptides results in alterations in the target living cell which provide the basis for separation of the altered cell. In turn, the gene or genes encoding the biologically active polypeptides can then be recovered via recovery of the altered cell.

In another preferred embodiment of the present invention, the stimulation (or inhibition) of a biological response by binding of displayed biologically active polypeptides results in the production (or cessation of production) of a molecule (herein termed the "modulator") from a target living cell which, in turn, leads to the recovery of genes encoding the biologically active polypeptides. The invention provides for 3 broad methods for recovery of genes encoding the biologically active polypeptides as follows;

(1) "tagging" - in this method, the modulator produced by the target living cell binds to the complex including the displayed polypeptide and thus tags this complex for subsequent isolation. For example, the target living cell may produce, as a result of the biologically active polypeptide, a protein or proteinaceous complex which binds to a ribosome complex displaying the biologically active polypeptide which includes the mRNA encoding it. The preferential binding of the modulator to the complex displaying the biologically active polypeptide would be as a result of the close proximity of this complex to the target living cell, for example at the surface of the target cell or within the target cell. Following production of the modulator by the target cell, the tagged complex including the displayed polypeptide can then be isolated, for example by using an antibody which binds to the modulator to separate the complex displaying the biologically active polypeptide from other complexes devoid of biologically active polypeptides. Following separation of these complexes, the genotype encoding the biologically active polypeptide can be determined. In addition to its application in ribosome display, the tagging method could also be used with polypeptide display methods employing live microorganisms; for example, the modulator could be used to tag bacteriophage for subsequent isolation of tagged phage and recovery of genes encoding biologically active polypeptides.

(2) "complementation" - in this method, the modulator produced by the target living cell is necessary for the subsequent viability of a living microorganism. For example, the target living cell may produce, as a result of the biologically active polypeptide, a modulator which is required to restore the infectivity of a defective bacteriophage displaying the biologically active polypeptide and thereby containing the gene encoding it. Upon restoration of infectivity, the bacteriophage can then propagate through the infected host in order to amplify the gene encoding the biologically active polypeptide. The preferential binding of the modulator to the bacteriophage displaying the biologically active polypeptide would be as a result of the close proximity of this complex to the target living cell, for example at the surface of the target cell or within the target cell. Following amplification of the bacteriophage, the genotype encoding the biologically active polypeptide can be determined. In addition to its application in bacteriophage display, the complementation method could also be used with polypeptide display methods employing live bacteria; for example, the modulator could be an antibiotic, a drug resistance enzyme/factor or an essential nutrient which, under appropriate selection conditions, allow for selective growth of bacteria in close proximity to the target living cell and thus amplification of the genotype encoding biologically active polypeptides.

It will be apparent to those skilled in the art that the modulator released from target living cells for the tagging or complementation methods could also provide the tagging or complementation moieties indirectly, for example by release of these from

a liposome or other molecular compartment via lysis of liposomes induced where the modulator is, for example, a phospholipase or, for certain lipopolysaccharide-based liposomes, a galactosidase. Amongst the possible tagging or complementing moieties released from liposomes or other molecular compartments could be nucleic acids or infectious microorganisms. Released nucleic acids could include synthetic oligonucleotides which can tag, by annealing, the mRNA or rRNA components of ribosome complexes including the displayed biologically active polypeptide. Released infectious microorganisms could include bacteriophage which could thus infect the bacterial cell displaying the biologically active polypeptide and provide one or more genes which lead to the subsequent viability of that cell and thus amplification of the genotype. The released modulator could also be an enzyme which effects a conversion of a substrate to a product which acts as the tagging or complementation moiety. It will also be apparent to those skilled in the art that the modulator could be constitutively released from target living cells where such release could be blocked by the biologically active polypeptide. It will also be obvious that other changes in a target living cell as a result of binding by a biologically active polypeptide could result in either tagging or complementation; for example, a biological change in a target living cell might lead to fusion of that cell with liposomes containing modulators which are thus released for interaction with the complex or microorganism containing the biologically active polypeptide.

In one embodiment of the present invention, a library of plasmids containing different genes are transcribed and translated into polypeptide molecules which are then tested for biological effect on mammalian cells. Specifically, the plasmid library could comprise a library of genes cloned downstream of a promotor for RNA polymerase to produce mRNA transcripts which are then translated in vitro. The translated polypeptide mixture with it's RNA still associated is then administered to cells and changes in the cellular phenotype are measured. Cells with the desired changed phenotype are then isolated and subjected to PCR using primers specific for the mRNA molecules still associated with the ribosome/polypeptide complex. In this manner, genes encoding polypeptides which induce the desired cellular phenotypic effect are amplified and can be sequenced, recloned into expression vectors or recloned into transcription vectors for subsequent re-testing of the proteins encoded by the genes. Through the use of mutagenesis strategies such as through use of error-prone PCR, proteins can thus be evolved through successive rounds of testing for induction of the required phenotype in order to generate polypeptides with the maximal biological activity.

As an alternative to plasmid vectors which are transcribed and mRNA translated, other methods may be used within the method of the present invention to produce polypeptides linked to a determinable genotype; such methods include bacteriophage vectors for display of proteins on the surface of bacteriophage particles and plasmid vectors which encode polypeptides fused to DNA binding polypeptides which cause the polypeptide to be tested to bind to the DNA encoding this polypeptide.

Having produced polypeptides linked to a determinable genotype, the present invention provides for a range of methods to induce a biological activity on target living cells whereby the genotype of the biological activity can be determined. One simple method is to detect biological activity by the appearance or disappearance of a

cell surface marker which can then be used as the basis for separation of biologically active cells from inactive cells; commonly, this would be achieved using an antibody which binds to the cell surface marker and then using a method to separate antibody-bound from non-bound cells. Such a method would be via binding of magnetic beads to the antibody (e.g. using a bead-associated anti-immunoglobulin) or via fluorescence-activated cell sorting (FACS) whereby the antibody (or a second antibody) is fluorescently labelled. Having isolated cells with the required biological activity, the corresponding genotype can then be determined either PCR-mediated amplification of nucleic acid associated with the test polypeptide or by propagation of the bacteriophage bound to the separated cells.

Another method of linking a biological activity to a genotype is to engineer target cells to produce (or cease to produce) one or tagging moieties which bind directly to the complex linking the test polypeptide to it's genotype. For example, cells might be engineered to produce a RNA binding polypeptide (such as HIV tat protein) which binds to the mRNA associated with a mRNA/ribosome/polypeptide complex. This RNA binding polypeptide might be linked to a promotor which is activated as part of the biological effect or might be produced as a fusion polypeptide associated with another polypeptide which is activated as part of the biological effect. Following cell lysis or other release of the binding of test polypeptides to biologically active cells, the mRNA could then be isolated using, for example, an antibody which binds to the tagging moiety. Cells might also be engineered to produce a bacteriophage polypeptide which could complement other polypeptides in a disabled phage thus rendering the phage infective.

In cases where biological activity is accompanied by the appearance or disappearance of a cell surface marker, another adaption of the present invention is to link this surface marker to the release or generation of a tagging or complementation moiety. This can be achieved, for example, by the use of an antibody-enzyme conjugates which bind to the cell surface marker and subsequently catalyse the release of a tagging or complementation moiety, for example by the enzymatic lysis of liposomes by phospholipase C (or beta-galactosidase for certain lipopolysaccharide containing liposomes) to release a variety of tagging or complementation moieties, or for example by the enzymatic conversion by horseradish peroxidase of biotinyl-tyramide to generate reactive biotin moieties.

It will be understood by those skilled in the art that, as an alternative to a surface antigen, an internally produced moiety could be used as the basis for selection whereby, following stimulation (or inhibition) of a biological response, cells are permeabilised and this moiety is either accessed by a binding protein such as an antibody or used for an enzymatic conversion in such a way that a tagging or complementation moiety is ultimately generated which can then combine to the polypeptide display complex.

The following example illustrates the invention but should not be considered to limit the scope of the invention:

Example 1:
As models of cell lines producing a modulator as a result of stimulation of a biological response, the lymphoblastoid cell line A431 (ECACC No 85090402, from ECACC, Porton Down, UK) and an EGFR$^-$ variant of HeLa (ECACC No 85060701) (selected using an anti-EGFR antibody conjugated to phospholipase C) were used for the experiments, in both cases using variants secreting the modulator protein HIV tat. Cells were grown in DMEM supplemented with 10%FBS. From both lines were derived recombinant cell lines secreting the tat protein as follows: A synthetic HIV tat gene was purchased from R and D Systems (Abingdon, UK) and PCR amplified using the following primers;

TATfor: 5'-CCG TAT CTC GAG ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT GTC CAC TCC GAA CCA GTC GAC CCT AGA CTG-3'
TATrev: 5'-GAA TTC GGA TCC TTA CTA TTC-3'

Tge resultant PCR product was digested with *Xho*I and *Eco*RI and subcloned into a *Xho*I and *Eco*RI-digested pCI-neo vector (Promega, Southampton, UK). The tat expression plasmid was transfected into A431 and EGFR$^-$ HeLa by electroporation using a Biorad GenePulser II (Biorad, Hemel Hempstead, UK) and selected in G418. Secretion of tat was confirmed by ELISA as follows: Anti-tat antibody was made using the tat peptide 37-72 which was conjugated via its N-terminal cysteine residue to KLH using MBS according to *Antibodies, A Laboratory Manual*, eds Harlow E. & Lane D. 10ug of the conjugate was used to immunise Balb/c mice as above and serum was collected and used at 1:100 dilution in ELISA experiments. A 10ug aliquot of antibody was applied to an Immulon 2 96-well microtitre plates (Dynatech, Chantilly, VA, USA) according to the manufacturer's instructions, samples of cellular supernatants were then added and incubated for 2 hours at room temperature, plates were then washed 3 times with PBS, and a 1:1000 dilution of HRP-labelled goat anti-mouse antibody conjugate (#A4416, Sigma, Poole, UK) was added and incubated for a further 1 hour at room temperature. After 3 washes in PBS, TMB substrate was added according to *Antibodies, A Laboratory Manual* ibid; this confirmed the presence of HIV tat protein in the culture supernatant of transfected but not untransfected A431 and EGFR$^-$ HeLa cells.

For model protein ligands, two single chain antibody (scFv) genes were used encoding anti-EGFR (epidermal growth factor receptor) (PCT00443) and anti-fluorescein (Denzin & Voss, Journal of Biological Chemistry, vol 267 (1992) p8925-8931). The anti-EGFR scFv was PCR amplified from cloned Vh and Vk chains using the following primers;

340Vhfor: 5'-CAG CTG CAG GAG TCT GGG GGA GGC TTA G-3'
340Vhbck: 5'-TCA GTA GAC GGT GAC CGA GGT TCC TTG ACC CCA GTA-3'
340Vkfor: 5'-GTG ACA TTG AGC TCA CAC AGT CTC CT-3'
340Vkbck: 5'-CAG CCC GTT TTA TCT CGA GCT TGG TCC-3'

PCR amplification reactions were performed using Expand High Fidelity PCR system (Boehringer, Lewes, UK). Reaction conditions for amplification of DNA fragments

were 1X Expand HF buffer, 2.5 mM $MgCl_2$, 4 mM of each dNTP, 2.5 units of polymerase, 10 ng template DNA and 30 pmol of primer DNA. Reactions were incubated in a thermal cycler using the following programme: 92°C for 5 min, 53-67°C (depending on primer sequence) for 5 min, 72 °C for 1 min, followed by 30 cycles of 92°C for 1 min, 53-67°C for 1 min and 72°C for 1 min. The resultant fragment was then purified using Wizard PCR purification columns (Promega). The PCR fragments of Vh and Vk were digested with *PstI/BstE*II and *SacI/XhoI* respectively. The scFv vector was pPM1His provided by Dr Peter Molly of Aberdeen University. This was digested with *PstI* and *BstE*II, the 3.5kb vector fragment was purified and the *PstI/BstE*II Vh PCR fragment was ligated and transformed into E.coli TG1 to produce a 340Vh recombinant. The 340Vh plasmid was next digested with *SacI* and *XhoI* and the *SacI/XhoI* Vk PCR fragment was cloned into the vector fragment to produce a 340 scFv-producing TG1 cells.

For cloning into a T7 polymerase transcription vector, the 340 scFv was amplified using forward primer 5'-CCG TAT AGA TCT ATG GAA GTG CAG CTG CAG GAG TCT GGG-3' and reverse primer 5'-CCG TAT GGA TCC TGC AGC CAC AGT CCG TTT GAT-3'. The PCR fragment was digested with *Bgl*II and *Bam*HI and cloned into the *E. coli* expression vector pET-9 (Promega) at the *Bam*HI site. This plasmid contains the promoter, translational start site and terminator from the bacteriophage T7 gene 10. The resultant plasmid was designated pEGFR1. The anti-fluorescein scFv DNA sequence was generated as described by Mallender, Carrero & Voss (Journal of Biological Chemistry, vol 271 (1996), p5338-5346) from the expression vector pGX8773 using forward primer, fox1 5'-CCG TAT AGA TCT ATG AAG TTG CCT GTT AGG TTG-3' and reverse primer, rox1 5'-CCG TAT GGA TCC TGA GGA GAC GGT GAC TGA GGT-3'. This fragment was generated by PCR and then cloned into the pET-9 vector as described above and designated pOX1.

A spacer sequence based on the glycine rich linkers of gene III of filamentous phage M13 was generated by performing PCR on a preparation of double stranded M13 DNA using the following two sets of primers:

m13f1: CCG TAT <u>AGA TCT</u> GGCTTTAATGAGGATCCATTC
*Bgl*II

m13r1: CCG TAT <u>CTC GAG</u> CTGTAGCGCGTTTTCATCGGC
*Xho*I

m13f2: CCG TAT <u>GTC GAC</u> GGCTTTAATGAGGATCCATTC
*Sal*I

m13r2: CCG TAT <u>TGA TCA</u> CTGTAGCGCGTTTTCATCGGC
*Bcl*I

Two sets of PCR reactions were performed using primer combination m13f1 and m13r1 or with m13f2 and m13r2. These two sets of reactions generated two populations of products, one with a 5' *Bgl*II and 3' *Xho*I and one with a 5' *Sal*I and 3'

*Bcl*I restriction sites. The restriction sites were included to facilitate the construction of multimers of the 30 amino acid linker. The *Bgl*II / *Xho*I PCR products were double digested and phosphatased and then ligated with the digested *Sal*I/*Bcl*I PCR products. In this way multimers ligated only 5' to 3' (which could be confirmed by digestion) would be formed. A 900 bp fragment was isolated by agarose gel electrophoresis and purified using Wizard PCR purification columns (Promega) according to manufacturers instructions. The fragment was then digested with *Bgl*II and *Bcl*I and cloned into the *Bam*HI site of pEGFR1 and pOX1 downstream of the scFv fragments to generate pEGFR2 and pOX2 respectively.

The HIV transactivation response element (TAR) sequence was inserted downstream from the M13 spacer segment by ligating the *Bcl*I digested 900 bp spacer fragment (generated as described above) to a self annealed oligonucleotides encoding the HIV TAR as follows:

TAR1: GATCAGCCAGATTTGAGCAGC
TAR2: GATCGCTGCTCAAATCTGGCT

The fragment was repurified and cloned into the *Bam*HI site of pEGFR2 and pOX2 to generate pEGFR3 and pOX3. Following cloning, sequencing of the PCR generated inserts was performed by the dideoxy chain termination method using a double-stranded plasmid DNA template (Kraft *et al.*, BioTechniques 6 (1988), p544) and Sequenase (Amersham) using T7 sequencing primers.

In vitro transcription of the constructs pEGFR3 and pOX3 was performed using the RiboMAX large scale RNA production system (Promega) according to the manufacturers instructions. The resultant mRNA was purified using PolyATtract system (Promega) and mixed in a 1:1 ratio (w/w).

In vitro translation was performed in an *E. coli* S-30 system as described by Chen and Zubay (ibid) modified as described by Hanes and Pluckthun (ibid). After 10 minutes of translation, a preparation of SRP (produced by the method of Romisch *et al.*, Nature 340, (1989), p478-482) was added and the translation continued for a further 10 minutes. The translation was stopped and the mixture centrifuged as described by Hanes and Pluckthun (ibid). The translation reactions were then diluted into PBS. Cells were grown up and harvested in trypsin/EDTA and washed twice in PBS before being resuspended DMEM/10%FBS at $2x10^6$ cells/ml either as a mixture of 10^6 A431 and 10^6/ml EGFR$^-$ HeLa cells or $2x10^6$/ml of each individual cell type. In each case, either tat$^+$ transformed or tat$^-$ untransformed cells were used. 100ul of cell suspension was then mixed with the translation reaction derived from an original 1ug of library DNA in 100ul BBS plus 0.1% sodium carboxymethylcellulose (ICI, Teeside, UK) at 37c for 1 hour. Cells were then centrifuged, washed twice in PBS containing 0.1% (v/w) BSA and resuspended in 100ul of this buffer.

Tat-bound mRNA was recovered by dissociation of mRNA in EDTA buffer as described by Hanes and Pluckthun (ibid) followed by passage through a column comprising polyclonal anti-tat antibody on protein A beads prepared according to *Antibodies, A Laboratory Manual* ibid. Washing and dissociation of retained ribosome complexes, isolation of mRNA, reverse-transcription PCR and repeated

transcription-translation were as described by Hanes and Pluckthun (ibid). After 2 rounds of ribosome display, the PCR products were cloned into pUC18 for sequencing and determination of the original scFv's in the selected population of genes. For this determination, the inserts in at least 50 pUC18 clones were partially sequenced in order for the identity of the clones to be determined. The results, as shown in table 1, indicate that the tat secreted from A431 cells provides a selective advantage to translated anti-EGFR mRNA over anti-fluorescein scFv which does not bind to A431 cells. Neither tat$^-$ nor EGFR$^-$ HeLa cells provide such a selective advantage showing that both binding to a target cell and tat secretion are required for selection.

	% EGFR scFv	
	tat⁻	**tat⁺ (transformed)**
A431	n/a	98
EGFR⁻ HeLa	29 (7 clones)	46
A431 + EGFR⁻ HeLa	64	100